A PROMOTER FOR HIGH-THROUGHPUT SCREENING FOR INHIBITORS AGAINST MYCOBACTERIA UNDER LOW CARBON CONDITIONS

This application claims benefit of Provisional Application No. 60/442,511 filed January 27, 2003; the disclosure of which is incorporated herein by reference.

FILED OF INVENTION

The present invention relates to a promoter for high-throughput screening for inhibitors against Mycobacteria under low carbon or starved conditions, more specifically promoter sequence of *rel A* gene for high-throughput screening for inhibitors against Mycobacteria under low carbon or starved conditions

BACKGROUND INFORMATION

Many bacteria can assume a well-defined physiological state under starvation, which facilitates their survival (Spector *et* al, 1988; Nystrome *et al.* 1989; Matin, A, 1991). The role of ppGpp in the developmental process of these physiological states has been a subject of interest for many researchers over the years. It has been extensively studied in *Myxococcus xanthus* where accumulation of ppGpp has been observed to be an important requirement for the formation of fruiting body (Harris *et al.* 1988). In *Streptomyces coelicolor*, ppGpp has been implicated in synthesis of antibiotics in the stationary phase of the bacteria (Chakraburty and Bibb, 1997) Though ppGpp has been detected in various other prokaryotes e.g. *Bacillus subtilis* (Ochi *et al.* 1982), *Bacillus stearothermophilus* (Fehr and Richter, 1981), *Staphylococci* (Cassesl *et al.* 1995), *Streptococcus equisimilis* (Mechold *et al.* 1996), *Salmonella typhimurium* (Kramer *et al.* 1988; Shand *et al.* 1989) under starvation, its function in these organisms is yet to be assigned.

Bacteria adapt to nutritional stress for their survival predominantly through a mechanism termed the stringent response. The hallmark of the stringent response is the accumulation of ppGpp, also called stringent factor, and down regulation of stable RNA (rRNA and tRNA) synthesis (Cashel *et al.*1996). It appears that RNA polymerase is the ultimate target of ppGpp (Chatterji *et al.* 1998), although the exact mode of selective down regulation of the gene expression is not clear.

Mycobacterium Smegmatis grown under carbon depletion condition serves as a best model of *Mycobacterium Tuberculosis* under latency towards drug screening.

Mycobacterium smegmatis is a fast growing counterpart of *M. tuberculosis* (M.tb), which is non-pathogenic in nature and thus easy to handle. Moreover, both these organism along with other mycobacteria share many of the characteristic features making them suitable model for each other.

Such common metabolic pathways leading to the survival of the organism have been known since sometime now. Extensive work to prove that latent *M.tb* can indeed be represented by *M.smegmatis* under depleted carbon source has been carried out and well known (Ojha et al., 2002). The studies by Ojha et al (2002) describe some of the recent observations to validate this model and establish that without these recent observations the present invention and model cannot be supported.

Although *Mycobacterium smegmatis* is non-pathogenic, it shares many biosynthetic pathways of *Mycobacterium tuberculosis* and may serve as a good model system. In addition, its faster growth rate makes it a suitable candidate for starvation studies. It has been shown that ppGpp accumulation is accompanied by morphological change in *M. smegmatis* under carbon starvation. Furthermore, *M. smegmatis* assumes the coccoid morphology (similar to the persistors) when ppGpp is ectopically produced by overexpression of *E.coli relA* in an enriched nutritional medium. It has also been characterised the *in vivo* function of *M.tuberculosis relA/spoT* homologue in *M.smegmatis* (Ojha et al, 2000).

The development of molecular genetic tools is needed to understand the mechanisms regulating gene expression in mycobacterial species.The slow growth rate of mycobacterial pathogens could be attributed to sluggish transcription initiation which in turn, perhaps, is due to the lower occurrence of strong promoters in mycobacterial genome. This is one of the reasons why a sufficiently strong and inducible expression system has not yet been established for mycobacteria. This can be achieved by providing a strong mycobacterial promoter upstream to the desired gene. With such a vector, the gene of interest, from a slow growing pathogen, can be successfully expressed in the heterologus faster growing mycobacterial species, which can act as a surrogate host.

Studies on the regulation of gene expression in any system are facilitated by simple and reliable assays, which can be quantitated and monitored both *in vitro* or *in vivo*. Reporter technology thus relies on fusing an assayable expression in both homologus and heterologous system, whose products are stable, with a promoter having sequence that can be regulated by different signals. Reporter genes have become convinient tools for studying mycobacteria and several such systems are known in the literature (Tyagi et al.,1997). Out of the many, few have become very popular and are widely used because of their control and inducibility (Stover et al., 1991; Parish et al., 1997). Recently *xylE* reporter assay has been proposed for high through-put screening in mycobacteria (Dastur and Varshney, 2001) and perhaps several such systems will be necessary in order to quantitate the relative strength of each assay against a target gene in mycobacteria.

By far the best candidate for reporter assay in *E.coli* has been the *lacZ* expression system where the *E.coli lacZ* gene encoding β-galactosidase (Fowler and Zabin, 1983) has been extensively used with various substrates like lactose or its derivatives to catalyze the cleavage of β-1,4 linkage producing galactose and glucose as products. One of the common derivatives of lactose has been ONPG (o-nitrophenyl-β-D-galactopyranoside), which yields a colored product and can be monitered spectrophotometrically (Miller, 1972). In addition, the presence of the chromogenic substrate X-gal (5-bromo-4-chloro-3-indolyl-D-galactopyranoside) in nutrient agar plates results in blue color in colonies expressing *lacZ* and thus the appearance of blue or

white colonies mark the presence of *lacZ* in solid media as opposed to ONPG assay in an aqueous environment (Timm et al., 1994a, 1994b; Bannantine et al., 1997; Jain et al., 1997). Varying degree of "blueness" in a colony, in principle can tell the relative strength of a promoter.

Several attempts have been made in the past to fuse a mycobacterial promoter sequence with *lacZ* with varying degree of success (Dellagostin et al., 1995; Knipfer et al., 1998; Kumar et al., 1998). One of the problem was the instability of *lacZ in M.smegmatis* due to transposition of an element IS 1096 and subsequent deletion of the vector (Cirillo et al., 1991; Chawla and Das Gupta, 1999).

The investigations by the inventors have shown a carbon starvation induced stringent response pathway in *M.smegmatis* (Ojha et al., 2000, Chatterji and Ojha., 2001, Ojha et al., 2002). The product of stringent response (p)ppGpp is maintained within the cell by two enzymes RelA and SpoT and in gram positive organisms like mycobacteria both the enzymes are part of a same gene known as *rel* (Ojha et al., 2000). An earlier work of the inventors have revealed the cloning and expression of 1.5 kb upstream fragment of *rel* from *M.tuberculosis* (Ojha et al., 2000). This gene expresses well and shows all its characteristics in the surrogate host *M.smegmatis* . In this present invention the inventors have identified a 200 base pair sequence upstream to the *rel* gene which when fused with *lacZ* shows stronger promoter activity than *hsp*60 promoter. The shows identification of an–10 promoter sequence by base specific mutation and observed that the plasmid bearing *lacZ* fused with 200base pair *rel* fragment is stable.

This promoter sequence of 200 bp is useful for high-throughput screening and developing novel inhibitors against Mycobacteria under low carbon or starved conditions. In other words use of this novel 200 bp promoter open new vistas and provides a new system that would enable the TB drug developers to isolate and develop highly efficient inhibitors or medicines against ever evolving and changing *M.tuberculosis* mycobacteria.

OBJECTS OF THE INVENTION

The main object of the invention relates to promoter for high-throughput screening for inhibitors against Mycobacteria under low carbon or starved conditions.

Yet another object of the present invention relates to a method of isolating a promoter for high-throughput screening for inhibitors against Mycobacteria under low carbon or starved conditions.

Still another object of the invention relates to use of promoter for high-throughput screening and developing inhibitors against Mycobacteria under low carbon or starved conditions.

One more object of the invention relates to prevention of mycobacteria survival due to activation of the promoter under low carbon or starved conditions by identifying efficient inhibitors.

BRIEF DESCRIPTION OF ACCOMPANYING DRAWINGS/FIGURES

Figure 1. The nucleotide sequence of 1.5 kb DNA fragment upstream of *M. tuberculosis* relA/spoT.

Figure 2. Constitutive activity of 1.5 kb DNA fragment when assayed using *XylE* reporter system.

Figure 3a. The promoter activity of 200 bp fragment immediately upstream to the start codon of *M. tuberculosis* relA/spoT. A) pSD5B (promoterless *lacZ*) B) pAN12(200bp *rel* promoter+*lacZ*).

Figure 3b. β-galactosidase assay showing a constitutive activity of $P_{relMt.}$ Cells harboring pAN12 were grown in carbon enriched medium (2% glucose) till OD_{600} of 0.7 and then transferred to 7H9 medium without any carbon source. Cells were taken out at different time intervals and their β-galactosidase activity was measured. The background activity of empty vector (pSD5B) was used as a reference.

Figure 4. *lacZ* expresssion by 200bp promoter region occurs in *M.smegmatis*, but not in *E.coli*. A) pAN12 transformed into *E.coli* B) pAN12 transformed into *M.smegmatis*

Figure 5a. Comparative analysis of the promoter strength of PrelMt and the P_{hsp60} on 7H9 agar containing X-gal, A) pSD5B (empty vector), B) pAN12, C) pMV261 (empty vector) D) pHspLac(pMV261+*lacZ*) transformed *M.smegmatis*.

Figure 5b. PrelMt. (in pAN12) is approximately 2.5 fold stronger than Phsp60 (in pHasplac). Both the promoters were cloned upstream of *lacZ* and their strength were compared by measuring the β-galactosidase activity of the cells transformed with promoter-reporter construct.

Figure 6. Binding of *M.smegmatis* RNA polymerase to pSAK12 as seen by Gel mobility shift assay.1) Lane Free DNA (pSAK12) 2) Lane 1:20 (pSAK12:RNA Polymerase) 3)Lane 1:30 (pSAK12: RNA Polymerase) 4)Lane 1:50 (pSAK12:RNA Polymerase) 5)Lane Free DNA (PGEM7Z) 6)Lane 1:20 (PGEM7Z:RNA polymerase) 7) Lane 1:30 (PGEM7Z:RNA polymerase) 8)Lane 1:50(PGEM7Z:RNA polymerase).

Figure 7. 8% denaturing PAGE of in vitro transcripts from pSAK12. Lane1.Molecular weight marker , Lane 2. PGEM7z (single round transcription) Lane 3. pSAK12 (single round transcription)) Lane 4. pSAK12 (multiple round transcription) Lane 5. pSAK12 + 50 μg rifampicin (single round transcription).

Figure 8. Mutagenesis of Three conserved T bases in putative –10 *rel* promoter region.

Figure 9. Effect of three mutations on *lacZ* expression in *M. smegmatis*.a) pSD5B b) pAN12 c) pSS12 d) pSS22 e) pSS32

SUMMARY OF THE INVENTION

Accordingly, the present invention relates to a promoter high-throughput screening for inhibitors against Mycobacteria under low carbon or starved conditions. Further, the use of this novel 200 bp promoter open new vistas and provides a new system that would

enable the TB drug developers to isolate and develop highly efficient inhibitors or medicines against ever evolving and changing *M.tuberculosis* mycobacteria.

DETAIL DESCRIPTION OF INVENTION

It has been observed that Mycobacteria have nature of recurring in patients who have been inflicted by attack of TB once. Many times the mycobacteria are also not completed eliminated by the medication. Further, mycobacteria has very fast degree of evolving themselves and thus they cannot be eliminated by presently available strong and multidrug therapy. Therefore, there is an imperative need to identifying and develop new drugs. However, the presently available systems are inadequate to address this problem.

There is no suitable model till today for drug screening against *Mycobacterium tuberculosis* (M.tb), which show long term persistence within host or known as latent M.tb. However, they are the major source of concern as actively growing or dividing species can be efficiently treated with major antibiotics.

Extreme slow growth rate of *M.tb* under latent stage makes it a very unfriendly candidate for high throughput screening. Clearly, a model for latency is warranted which should show comparatively fast growth rate, which can be manipulated with ease.

In the present study mycobacteria *M.smegmatis* which is fast growing and non-pathogenic, but a very close counter-part of *M.tuberculosis* has been used as a model system for identifying a novel promoter which allows the mycobacteria to survive under low carbon or starved conditions and escape the multidrug therapy during treatment. The identified novel promoter has been identified to serve as better system in developing the new and efficient inhibitors against the mycobacteria. This novel isolated promoter is better and more efficient than the known promoters being used for identifying new and efficient inhibitors or drugs.

In the present invention the it has been shown that the 200bp upstream fragment obtained from 1.5kb *rel* promoter of *M.tuberculosis* is sufficient for promoter activity and is constitutive in nature. The promoter is stronger in comparison to hsp60 promoter. Such a simple *blue/white* selection and promoter specificity for mycobacterial RNA polymerase, would go a long way for both quantitative and qualitative assessment of the mycobacterial promoter strength. In addition, any gene cloned downstream of *rel* promoter in correct orientation would show good expression, expectedly. The stability of the plasmid for a considerable length of time is an added advantage. Although we expected a regulatory, starvation controlled promoter element from a upstream sequence of the *rel* gene, even the 1.5kb upstream promoter sequence showed constitutive expression with *xylE* gene (Fig .1) and thus we did not persue this point further. There could be other regulatory elements which cannot be detected by the assay presented here. This this system would find a wide range of application. The single round heparin-resistant transcription by *M.smegmatis* RNA polymerase reported here has not been reported in the literature earlier. Therefore in the present invention the reconstituted transcription machinery would help to dissect the mechanism of transcription regulation in mycobacteria and also show that the promoter is responsible for such functions i.e this specific promoter is activated only under the stress conditions which further on activates the RNA polymerase activity thereby allowing the mycobacteria to survive under the low carbon or starved conditions. Reconstitution of RNA polymerase from individual subunit will be an added advantage. Further, the use of this novel 200 bp promoter open new vistas and provides a new system that would enable the TB drug developers to isolate and develop highly efficient inhibitors or medicines against ever evolving and changing *M.tuberculosis* mycobacteria.

In order to delineate the control of gene expression in Mycobacteria, one has to have an efficient reconstituted expression system and reconstituted RNA polymerase, the enzyme responsible of gene expression. A US patent No. 6,355,464 has already been granted towards the second goal i.e., reconstitution of RNA polymerase from *M.tuberculosis*. In this patent it has been reported the high throughput screening of natural inhibitors against this reconstituted enzyme. However, their method fails to use an easy, high stringent

assay of RNA polymerase or gene expression for this screening purpose. On the other hand, a detectable gene expression system can pinpoint the effect of inhibitors on RNA polymerase based assay by looking at the level of expression. Moreover, a battery of inhibitors can be studied by varying degree of response at different genes. The present study of a simple *lacZ* expression system used with *rel* promoters showed that this promoter is very efficient and thus can be engineered with any unknown open reading frame and then can be studied for their expressibility by transcribing them with mycobacterial RNA polymerase.

Accordingly, the main embodiment of the present invention relates to a promoter having a SEQ ID No.2 for high throughput screening and developing inhibitors of mycobacteria under low carbon source or starved conditions.

Yet another embodiment of the present invention relates to the a promoter wherein the promoter is 2.5 folds more active than the conventional P_{hsp60}(heat shock protein expression system).

Another embodiment of the presenting invention relates to the a expression system for high-throughput screening and developing inhibitors of mycobacteria under low carbon source or starved conditions said system comprising of a promoter of 200 bp having sequence ID No.2 in a vector pSAK12.

One more embodiment of the present invention relates to a method of preparing a promoter expression system for high-throughput screening and developing inhibitors of mycobacteria under low carbon source, said process comprising the steps of:

(a) isolating and characterizing a 200 bp promoter sequence having SEQ ID No.2 from nucleotide sequence of relA/spoT of *M.smegmatis* having a SEQ ID No. 1,

(b) ligating the isolated promoter sequence of step (a) in vectpr pSAK12, and

(c) studying the expression of the promoter sequence under low carbon source or carbon starved conditions.

Still another embodiment of the present invention relates to the wherein carbon source glucose is in the range of about 2.5 -0.001%.

One more embodiment of the present invention relates to the carbon source, glucose is in the range of about 2 to 0.02%.

Another embodiment of the present invention relates to the percentage inhibition growth of bacteria in presence of the promoter and the inhibitor ethambutol is reduced in the range of about by about 6 to 25 % in presence of 0.02% glucose i.e under starved conditions.

Still another embodiment of the present invention relates to the percentage inhibition growth of bacteria in presence of the promoter and in presence of inhibitor ethambutol is reduced in the range of about by about 7 to 21 % in presence of 0.02% glucose i.e under starved conditions.

One more embodiment of the present invention relates to the percentage inhibition growth of bacteria in presence of the promoter and the inhibitor Isoniazide is reduced in the range of about by about 15 to 45 % in presence of 0.02% glucose i.e under starved conditions.

Another embodiment of the present invention relates to the wherein percentage inhibition growth of bacteria in presence of the promoter and the inhibitor Isoniazide is reduced in the range of about by about 18 to 40 % in presence of 0.02% glucose i.e under starved conditions.

Still another embodiment of the present tin invention relates to the wherein percentage inhibition growth of bacteria in presence of the promoter and the inhibitor Rifampicin is reduced in the range of about by about 20 to 45 % in presence of 0.02% glucose i.e under starved conditions.

Yet another embodiment of the present invention relates to the percentage inhibition growth of bacteria in presence of the promoter and the inhibitor Rifampicin is reduced in the range of about by about 21 to 41 % in presence of 0.02% glucose i.e under starved conditions.

The following examples are given by way of illustration of the present invention and therefore should not be construed to limit the scope of the present invention.

EXAMPLES

EXAMPLE 1

Bacterial strains, medium and growth condition

All the plasmids used in this study are enlisted in table1. *Mycobacterium smegmatis*, mc^2155, was used in all the experiments. The bacteria were grown in 7H9 medium supplemented with 2% glucose, 0.05% Tween-80 and 25μg/ml kanamycin, unless mentioned otherwise. For plate culture, 1.5% agar was added to the liquid medium. For plate assay of *lacZ*, bacteria were grown in 7H9 plate containing 40μg/ml of X-gal. The *E.coli* strains were maintained in LB or LB agar with either 50μg/ml of kanamycin or 100μg/ml of ampicillin.

EXAMPLE 2

Transcriptional fusion of *M.tuberculosis relA/spoT* to *xylE* reporter.

The BamHI-SphI fragment which contained 1.5kb upstream to start codon of *M. tuberculosis relA/spoT* was taken out from the cosmid MTCY227 (a gift from S.T.Cole: Cole et.al. 1998) and subcloned in the BamHI-SphI site of pTZ19U (Bio-Rad). Then the fragment was released by KpnI-BglII site and cloned into KpnI-BamHI site of pTKmx (Kenney and Churchward, 1996). The resulting recombinant plasmid, pAKO1, had the *xylE* reporter transcriptionally fused to the 221^{st} nucleotide of *relA/spoT* gene (Table 1).

EXAMPLE 3

Measurement of transcriptional activty of the sequence upstream to *relA/spoT*:

The *M. smegmatis* , mc^2155, transformed with pAKO1 was cultured till the mid-log phase (OD_{600} = 0.7) in 7H9 medium (with 2% glucose, 0.05% Tween-80 and 25μg/ml kanamycin) and then harvested, washed and transferred to 7H9 medium containing either 2% glucose, 0.2% glucose, or, 0.02% glucose and assayed for xylenase activity at different time intervals. The xylenase assay was carried out as described previously (Kenney and Charchward, 1996). Briefly, cells from 1ml culture were harvested, resuspended in 50μl of PBS and then10μl of cell suspension was added to 990μl of 0.5mM of Catechol. The reaction mix was incubated at room temperature for 10 minutes and then OD_{375} was obtained. The OD contributed by scattering of cells was also measured at 375nm. The activity/unit OD was calculated from the formula (Dastur and Varshney, 2001).

$$\text{Activity/unit OD} = \frac{[OD_{375}\text{ (reaction mix)} - OD_{375}\text{ (cell density)}]}{[OD_{375}\text{ (cell density)}]}$$

Cells transformed with pTKmx were used as a negative control for the assay. The activities obtained for pTKmx was subtracted from the activities obtained for pAKO1 (Table 1).

EXAMPLE 4

Cloning and characterization of 200bp upstream sequence proximal to the start codon of *relA/spoT* .

A set of two primers sak1 (CGGCCACGTTCGGTACCTCCGACCTAGA) and sak2 (GCCGTGTCGTGAGAATTCACGACGTGTTAG) were used to amplify the 200bp immediately upstream to *relA/spoT* (see fig1) from pAKO1. The PCR conditions were 94^0C for 1min., 66^0C for 30 sec and 72^0C for 30 sec. The 200bp amplicon was subcloned into pGEM-T Easy (Promega) to form pSAK12. The vector pGEM-T Easy is a linear vector with a single T overhang on either arm, which is flanked by multiple cloning sites. The linear vector with T overhang ligates to any PCR product which has A at the terminals (invariably added as a last base when Taq DNA polymerase is used in PCR). The clone with the correct orientation (the end proximal to the gene was towards SphI site) was picked and the 200bp insert was released by SphI-SpeI and ligated to SphI-XbaI ends of pSD5B (Jain et.al. 1997) to form a recombinant plasmid pAN12. pSD5B is a mycobacteria-*E. coli* shuttle vector with a promoterless *lacZ*. The promoter activity of the 200bp fragment was analyzed by assaying the *lacZ* activity of the *M. smegmatis* transformed with pAN12. The *lacZ* activity was assayed on plate as well as liquid culture as published earlier (Miller, 1972). *M. smegmatis* transformed with pSD5B was used as negative control.

For a comparative analysis between the promoter strength of 200bp fragment and the P_{hsp60}, the fragment containing *lacZ* was released from pSD5B by PstI digestion and

ligated to pMV261 (Stover et.al., 1991) at PstI site and screened for the correct orietation. The recombinant plasmid in correct orientation, pHsplac, in which *lacZ* was cloned in the direction of the Phsp60 was screened for further use. The *lacZ* activity of *M. smegmatis* cells transformed with pHsplac was compared with that of the cells harboring pAN12 in plate as well in liquid culture. The stability of pAN12 in the host strain, both *M.smegmatis* and *E.coli* was further checked by repeated subculturing for 10 generation, expressing *lacZ* gene on X-gal containing plate. Restriction anaylsis revealed that there is no addition or deletion of the sequence in the plasmid (Table 1).

Table.1

Catalogue of all the plasmids used

Plasmid	Size(bp)	Marker	Description
pTKmx	5998	Kan^R	pTKmx is a shuttle vector containing promoterless *xylE* gene
PAKO1	6208	Kan^R	pTKmx with 1.5kb DNA fragment, upstream to start codon of *M.tuberculosis* relA/spoT, cloned upstream of *xylE* gene
PGEMT Easy	3010	Amp^R	PGEMT Easy vector (supplied from promega)
PGEM7Z	2998	Amp^R	Same as PGEMT Easy vector (supplied from promega)
pSAK12	3231	Amp^R	PGEMT Easy vector with 200bp DNA fragment,upstream to start codon of *M.tuberculosis* relA/spoT, cloned upstream of *lacZ* gene
pSD5B	9500	Kan^R	pSD5B is ashuttle vector containing promoterless *lacZ* gene
pAN12	9760	Kan^R	pSD5B with 200bp DNA fragment, upstream to start codon of *M.tuberculosis* relA/spoT, cloned upstream of *lacZ* gene
pSS12	9760	Kan^R	1 st 'T ' of –10 region of promoter mutated to 'G' in pAN12
pSS22	9760	Kan^R	2 nd 'T ' of –10 region of promoter mutated to 'G' in pAN12

PSS32	9760	Kan^R	3 rd 'T ' of −10 region of promoter mutated to 'G' in pAN12

EXAMPLE 5

Gel retardation and single round transcription with *M.smegmatis* RNA polymerase

RNA polymerase from mid-log phase cells of mc^2155, *M.smegmatis* was purified according to the known protocol (Burgess and Jendrisak, 1975) mainly following the established method for purification of the *E.coli* enzyme. The purified enzyme shows full complementation of all the subunits ($\alpha_2\beta\beta^1\omega$) and two sigma subunits (σ^A and σ^B) (not shown).

0.1pmole of DNA template pSAK12 was incubated with 20,30 and 50 fold molar excess of *M.smegmatis* RNA polymerase in the presence of a buffer containing 50mM Potassium glutamate, 250mM Tris-HCl (pH 7.8), 15mM Magnesium acetate, 0.5mM Dithiothreitol, 0.5mM EDTA, 250μg/ml Bovine serum albumin and 25% glycerol. The incubation was carried out for 30 minutes at 37°C.The bound and unbound form of DNA was resolved on a 0.7% Agarose gel against 1X TBE buffer.

EXAMPLE 6

In vitro Transcription Assay

0.2pmole of linearized form of pSAK12 and 2pmole of *M.smegmatis* RNA polymerase were mixed in transcriptional buffer containing 500mM Tris-HCl(pH 7.8), 30mM Magnesium acetate,1mM EDTA,1mM DTT,500mM NaCl 300μg/ml BSA in a final volume of 35μl and incubated at 37°C for 45 minutes.The reactions were started by adding 15μl of prewarmed substrate-heparin mixture which contained 1.5μl of 10X transcriptional buffer, 2μl of 25X NTP mixture(4mM each of ATP,CTP,GTP,1.25mM UTP and 2μCi of α^{32}p UTP(3000cimmol^{-1})and 2μl of 5mg/ml of heparin (sodium salt). The reaction was allowed to proceed for 15 minutes at 37°C and stopped by addition of 50μl of a stop solution containing 40mM EDTA and 300μg/ml yeast tRNA. Transcriptional product was precipitated overnight at - 20°C by adding 1/10th volume of 3M sodium acetate (pH5.2) and 2.5 volume of 100% ethanol. The precipitate was washed with 70% ethanol and dried, dissolved in 15 μl of deionized formamide loading dye,

heated to 90^0C for 5 Minutes and cooled on ice. The precipitate was loaded on a 8% denaturing polyacrylamide gel containing 7M urea and run in 1X TBE at constant 250 volt. The gel was dried and exposed to X-ray films for 24 hours at -70^0C. For multiple round transcription, reaction was carried out in the same way as the single round transcription excluding the addition of heparin. In order to study the inhibition of single round transcription, reaction was carried out in the presence of 50μg of rifampicin (Table 2). This *in vitro* study highlights the gist and aim of the experiment, wherein the inventors have used stationary phase or starvation induced promoter in expression vector pSAK12. The novel promoter like this can be assayed for inhibition of transcription activity and thus indirectly reflects the growth of the organism in presence of antibiotics or inhibitors. This very assay demonstrates that due to the activation of this promoter under starved or low carbon source conditions there is higher transcription thereby enhancing the percentage survival of mycobacteria. In other words in presence of classical inhibitors for example rifampicin on normal promoters inhibit only 50 to 70 percent inhibition of growth, whereas the same inhibition is reduced by about 20% in presence of the promoter of the present invention under stationary or starved state.

Table 2

Drug	**Concentration (μgm/ml)**	**% inhibition of growth at ***	
		2% Glucose	**0.02% Glucose**
Ethambutol	0.15	44	37
	0.31	85	66
	0.63	91	68
	1.25	93	72
Isoniazide	0.8	33	12
	1.6	70	30
	3.2	90	70
	6.4	96	78
Rifampicin	0.25	39	9
	0.5	58	17
	1.0	70	29
	2.0	89	68

* Percent inhibition growth at each point is the relative decrease in the optical density of liquid culture as compared to the control (without antibiotic).

EXAMPLE 7

Mutation of the promoter element

Site-specific mutagenesis was carried out by the *quickchange* protocol (Stratagene) in the –10 region of the promoter (TATCCT). The three highly conserved T bases in the –10 region of the promoter were mutated to either G or C bases. The PCR conditions were 94^0C for 3min, 65^0C for 30 sec and 72^0C for 3min, using pSAK12 as template. The mutations were confirmed by sequencing of the DNA. 200bp inserts both wild type and mutants were released by SphI-SpeI of pSAK12 and ligated to SphI-XbaI ends of pSD5B (Jain et al. 1997) to form pSS12, pSS22, pSS32. The strategy of molecular cloning was followed according to Sambrook et.al., 1989. The electroporation of *M.smegmatis* was carried out in cell electroporator (BTX) with 2mm-gap cuvette at 1.25kV/mm.

EXAMPLE 8

The 1.5kb DNA fragment upstream of *relA/spoT* ORF has a constitutive promoter activity.

The 1.5kb DNA fragment upstream of *relA/spoT* showed promoter activity when cloned in *xylE* reporter system (Kenney and Churchward, 1996) on pAKO1 (Fig 2). Surprisingly, the activity was constititutive with negligible change when shifted to a carbon starved medium. Moreover, there was a very strong level of expression even in carbon enriched culture. As 1.5 kb fragment was too big for promoter analysis, a search for promoter element nearest to *relA/spoT* was carried out using nested PCR.

EXAMPLE 9

The promoter activity of the large fragment was contained in a 200bp sequence immediately upstream to *relA/spoT*.

With a set of two primers, sak1 and sak2, a 200bp DNA fragment upstream to *relA/spoT* was amplified and cloned ahead of *lacZ* reporter system (Jain et.al. 1997) to form promoter-reporter construct on pAN12. Fig 3a shows that 200bp fragment was sufficient to produce the promoter activity which appears to be similar in strength to that of entire 1.5 Kb. A quantitative analysis of the promoter-*lacZ* system in liquid culture (Fig 3b) corroborated the data obtained with plate culture. Consistent with the promoter activity of 1.5 kb with *xylE* reporter, the activity of 200 bp was observed to be constitutive with a high level of expression even under nutrient enriched condition at zero time point. In

carbon starved conition, not additional increase in β-galactosidase activity was noticed. Although a set of nested PCR products with increment of 200 bp were also amplified, they were not analyzed further since the entire promoter activity was observed in the proximal 200 bp fragment. For further work we have referred this fragment as P_{relMt}.

Interestingly, the promoter activity of 200bp fragment was specific to mycobacteria and was completely lost in *E.coli* (Fig 4). This observation was consistent with the general property of most of the *M. tuberculosis* promoters that they are not active in *E.coli* (Dasgupta *et.al.*, 1993; for review see Mulder *et.al.*, 1997).

EXAMPLE 10

Promoter activity of P_{relMt} was stronger than P_{hsp60} and promoter directed transcription

As the promoter was constitutive and had a high basal level of expression, we compared the activity of this promoter with a widely used mycobacterial *hsp60* promoter (Fig. 5a). P_{hsp60} is one of the most common mycobacterial promoters used for *in vivo* gene expression (Stover et.al., 1991) and *in vitro* transcription (Levin and Hatfull, 1993). The strength of the promoter was measured as a direct function of activity produced by the promoter fragment. For comparative analysis, *lacZ* was cloned downstream to P_{hsp60} in pMV261 (Stover et.al., 1991). As the two promoter-reporter constructs were different, the final *lacZ* activities from the two constructs, P_{hsp60} - *lacZ* and P_{relMt} - *lacZ,* were obtained as the percentage increase in the activity due to the presence of the promoter. It was calculated as:

$$\text{Percentage increase in specific activity} = \frac{\text{sp.act. } (lacZ + \text{promoter}) - \text{sp.act. (empty vector)}}{\text{sp.act. (empty vector)}}$$

Fig 5 a & b show that P_{hsp60} was at least 2.5 fold less active as compared to P_{relMt}. This observation suggested that P_{relMt} might be a better template for developing a mycobacterial *in vitro* transcription system. Thus, we explored whether P_{relMt} can be used as a template for *in vitro* transcription.

EXAMPLE 11

M.smegmatis *RNA polymerase binds to pSAK12 : Promoter directed Transcription*

Fig.6 shows the electrophoretic mobility shift assay of pSAK12 with *M.smegmatis* RNA polymerase at varying molar ratio. It can be seen from the figure that the vector without 200 base pair *rel* promoter sequence, cannot bind the enzyme at any concentration (lanes 6-8) where as pSAK12 shows appreciable protein concentration dependent mobility shift with RNA polymerase (lanes 2-4).

As we noticed a very specific influence of 200bp upstream region of *rel* promoter in RNA polymerase recognition from previous experiment, it was thought that the promoter specific transcription reaction can also be detected (fig.7). It can be seen from lanes 3 and 4 that a short transcript was generated (≈ 37 bp) both in single and multiple round transcription which was rifampicin sensitive (lane 5).

EXAMPLE 12

Detection of promoter element

Putative mycobacterial sequences, published by Mulder et al., (1997), showed the *M.tuberculosis* promoter consists of a –10 consensus sequence TAyGAT(y-pyrimidine). Putative –10 consensus TATCCT sequence were identified in the 200bp promoter region of *rel*. The putative –10 consensus sequence of *rel* promoter are highly conserved at four positions as that of –10 consensus sequence. Fig .8 shows varying degree of conserved T base in the –10 promoter sequence. Thus we mutated the 1st position T base to G position, 3rd position T to G position, 6th position T to C position and studied their effect on *lacZ* expression. Fig .9 shows that the third T base which is 100% conserved had the maximum effect on *lacZ* expression as expected.

ADVANTAGE OVER EXISTING METHODS :-

In order to delineate the control of gene expression in Mycobacteria, one has to have an efficient reconstituted expression system and reconstituted RNA polymerase, the enzyme responsible of gene expression. An US patent (US 6,355,464 B1, dated March 12, 2002) has already been granted towards the second goal i.e., reconstitution of RNA polymerase from *M.tuberculosis*. They have reported the high throughput screening of natural inhibitors against this reconstituted enzyme. However, their method fails to use an easy, high stringent assay of RNA polymerase or gene expression for this screening purpose. On the other hand, a detectable gene expression system can pinpoint the effect of inhibitors on RNA polymerase based assay by looking at the level of expression. Moreover, a battery of inhibitors can be studied by varying degree of response at different genes. Our simple *lacZ* expression system used with *rel* promoters showed that this promoter is very efficient and thus can be engineered with any unknown open reading frame and then can be studied for their expressibility by transcribing them with mycobacterial RNA polymerase.

The two well known expression system used world-wide for mycobacteria are BCG heat shock induced promoter hsp60 and the other is acetamide inducible system. We have shown clearly that at least in one case (*hsp60*) our promoter (rel) is much better and we have estimated in quantitative terms the degree of difference between them.